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TOXICOLOGY STUDIES OF LEWISITE AND SULFUR MUSTARD AGENTS:  
GENETIC TOXICITY OF LEWISITE (L) IN CHINESE HAMSTER OVARY CELLS

FINAL REPORT

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## EXECUTIVE SUMMARY

Chemical warfare agents present an obvious risk to individuals suffering acute exposure, but they may also present long-term environmental or occupational health hazards for workers in operations involving these chemical agents. Occupational health standards have not been established for Lewisite (L) [dichloro(2-chlorovinyl)arsine] a potent vesicant which reacts with sulfhydryl groups of proteins through its arsenic group. Lewisite is used in a number of research laboratories, stored in depot sites throughout the country and occasionally transported to distant sites. The destruction of current stockpiles of Lewisite by the U.S. Army in the near future could create additional environmental and occupational risk. To establish a data-base for setting environmental and occupational standards, we conducted studies to evaluate the toxicity, mutagenicity, and reproductive effects of Lewisite using in vitro and in vivo study systems.

The cytotoxic, clastogenic mutagenic effects of Lewisite in Chinese hamster ovary cells were investigated and are described in this report. One mutation assay and two cytogenetic assays were used in this study. The mutation assay utilized the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus (6-thioguanine resistance). The two cytogenetic analyses were chromosomal aberration analysis, measurement of chromosome damage, and sister chromatid exchange (SCE), a measurement of chromosome rearrangement.

The CHO cells were exposed in the test system for 1 hour, then washed and cultured for an additional 20-30 hours, depending on the assay to be used. The total number of mutant colonies were determined and the mutation frequency was calculated. Chromosome aberrations were scored using 100 metaphases per dose and SCE per cell were calculated. (SDW) ←

One hour exposures to Lewisite were cytotoxic in  $\mu\text{M}$  amounts. The cell survival response yielded a  $\text{D}_{50}$  of 0.5  $\mu\text{M}$  and an extrapolation number of 2.5. The mutagenic response at the HGPRT locus was sporadic and not significantly greater than control values when cells were exposed over a range of 0.12 to 2.0  $\mu\text{M}$ . Sister chromatid exchange (SCE) induction, a measure of chromosomal rearrangement, was weakly positive over a range of 0.25 to 1.0  $\mu\text{M}$  but the values were not significantly greater than the control response. Chromosomal aberrations were induced at 0.50, 0.75 and 1.0  $\mu\text{M}$  in one experiment and 0.50

and 0.75  $\mu$ M in another experiment. The induced values were significantly greater than the control values. Lewisite appeared to be cytotoxic and clastogenic in our investigations but SCE and mutation at the HGPRT locus were not significantly greater than control values. Lewisite toxicity was in some ways similar to radiomimetic chemicals such as bleomycin.

## TABLE OF CONTENTS

	Page
FOREWORD . . . . .	1
EXECUTIVE SUMMARY. . . . .	3
INTRODUCTION . . . . .	7
MATERIALS AND METHODS. . . . .	11
Lewisite. . . . .	11
Chemicals Used. . . . .	12
Cell Culture Media. . . . .	13
Identification of Cell Line . . . . .	13
<i>In Vitro</i> Assays . . . . .	13
Mutation Analysis . . . . .	14
Cytogenetic Analysis. . . . .	14
Statistical Analysis. . . . .	15
RESULTS. . . . .	17
Cytotoxicity. . . . .	17
Mutation Results. . . . .	17
Sister Chromatid Exchange . . . . .	17
Clastogenic Effects . . . . .	22
DISCUSSION . . . . .	25
LITERATURE CITED . . . . .	27
PERSONNEL LIST . . . . .	29
QUALITY ASSURANCE STATEMENT. . . . .	30
DISTRIBUTION . . . . .	31

## LIST OF FIGURES

Figure		Page
1	Fraction of cells surviving Lewisite treatment . . . . .	18
2	Fraction of cells surviving Lewisite treatment in the presence of S9 . . . . .	19

# LIST OF TABLES

Table		Page
1	Relevant Chemical and Physical Data for Lewisite, Dichloro(2-chlorovinyl)arsine. . . . .	8
2	Lewisite Induced Mutations at the HGPRT Locus in CHO Cells .	20
3	Lewisite Induced Sister Chromatid Exchange in CHO Cells. . .	21
4	Lewisite Induced Chromosome Abberations in CHO Cells . . . .	23

## INTRODUCTION

Chemical warfare agents present an obvious risk to individuals suffering acute exposure but they may also present long-term environmental or occupational health hazards for workers in operations involving these chemical agents. Lewisite [dichloro(2-chlorovinyl)arsine], one of two major vesicant agents, presents a potential for accidental or occupational exposure because it is used in a number of research laboratories, stored in depot sites throughout the country and occasionally transported to distant sites. In addition, stockpiles of Lewisite are scheduled for destruction by the U.S. Army in the near future, creating an additional potential for environmental and occupational exposure. Although considerable information is known concerning the acute effects of Lewisite, few data are available on its long-term hazards. Segments of the population that may be particularly sensitive to its toxicity include the chronically ill, the young and old, and the unborn. It is this concern that has prompted these studies to identify the potentially toxic, mutagenic and reproductive effects of Lewisite and to establish a database for the development of hazard evaluations and occupational health standards for this chemical.

Lewisite is a highly toxic chemical vesicant. Unlike the strong alkylating vesicant sulfur mustard, Lewisite reacts with the sulfhydryl groups of proteins through its arsenic group (Cassarett and Doull, 1986). In the presence of water or alkalis, Lewisite hydrolyzes to form Lewisite oxide, which is non-volatile and insoluble in water. Although few data are available, Lewisite oxide is generally thought to be a weaker vesicant (Gates et al., 1946) but its toxicity has yet to be determined. Relevant chemical and physical data for Lewisite are summarized in Table 1.

A comprehensive review which summarized the chemical and toxicity data of Lewisite acquired during World War I and World War II was published in 1946 (Gates et al., 1946). This review compared known human and animal data and concluded that sufficient toxicologic data were available for the determination of military usage. Lewisite exposure is characterized by immediate onset of pain, unlike the action of sulfur mustard in which pain may be delayed. The mucus membranes of the respiratory and gastrointestinal

TABLE 1. Relevant Chemical and Physical Data for Lewisite,  
Dichloro(2-chlorovinyl)arsine<sup>a</sup>

Cas #:	541-25-3
RTECS #:	CH2975000
Structural formula:	$\text{Cl}-\text{CH}=\text{CH}-\text{AsCl}_2$
Molecular weight:	207.3 g
Density at 20°C:	1.888 g/ml
State:	Dark, oily liquid (stable in steel and glass)
Vapor pressure at 20°C:	0.394 mm
Decomposition temperature:	>100°C
Solubility in water:	Very slightly soluble
Hydrolysis	
Rate:	Rapid
Products:	Chlorovinyl arsenous oxide, HCl (in acid solutions)
	Acetylene, sodium arsenate (in alkaline solutions)

<sup>a</sup>Rosenblatt et al. 1975

tracts are particularly sensitive to Lewisite damage. Lewisite is not only a lethal vesicant but is also a systemic toxin; the liver, kidneys, gall bladder, bile duct and other organ systems are vulnerable to damage if absorption occurs (Cameron et al. 1946).

Exposures to Lewisite vapor produces edema of the respiratory tract and accumulation of pleural fluid (Gates, et al., 1946). Skin lesions resulting from contact with liquid Lewisite involve the rapid formation of an erythematous area, subsequent vesication and penetration of subcutaneous tissue so that edema and necrosis are evident. Man was less sensitive to skin lesion induction than the dog or rabbit. Systemic intoxication was evident in the dog a few hours following application of Lewisite (Gates et al., 1946). Although sufficient anatomical lesions to characterize the immediate cause of death were not apparent, it was reported that fluid losses due to changes in capillary permeability did cause remarkable decreases in blood volume. Comparisons of toxic effects of Lewisite and sulfur mustard in dogs and rabbits indicated that Lewisite was more damaging to the skin and was more likely to induce systemic poisoning than was sulfur mustard.

Few data are available to evaluate the potential chronic effects of Lewisite other than information based on anecdotal evidence from war use. based on one incidence of accidental exposure to a soldier's leg, Lewisite is considered a suspect carcinogen in man (Krause and Grussendorf, 1978). Workers of a Japanese factory producing mustard and Lewisite agents during World War II had a high mortality rate due to respiratory and gastrointestinal cancers (Wada et al., 1968; Yamakido et al., 1985). These workers were potentially exposed to unknown quantities of both sulfur mustard and Lewisite; therefore, it is not possible to implicate Lewisite as a carcinogen because of possible confounding effects of the carcinogen sulfur mustard.

Virtually no data were found on the mutagenicity of Lewisite in the literature. Auerbach (1947) found no mutagenic response in the fruit fly exposed to Lewisite and Loveless (1951) reported normal cellular division in root tips exposed to aqueous solutions of Lewisite. The teratogenic potential of Lewisite was studied by Hackett et al. (1987) in rats and rabbits using a segment II teratology protocol. Rats were exposed to 0.5, 1.0 or 1.5 mg/kg Lewisite via gastric intubation from 6 to 15 days of gestation (dg) and fetuses were examined on dg 20. No evidence of a teratogenic response to Lewisite was observed. Likewise, fetal development of the rabbit exposed to 0.07 to 0.6 mg/kg Lewisite between 6 and 19 dg was not affected even though maternal mortality was induced. These results suggest that Lewisite is not teratogenic in the rat or the rabbit after short term exposures since fetal effects were observed only at dose levels that induced maternal toxicity.

It is of interest that many of the symptoms of Lewisite and arsenic intoxication are similar (severe inflammation of the gastrointestinal tract with electrolyte disturbances and ulceration and perforation of membranes) (NAS, 1977) and raise the possibility that the toxicity of Lewisite may result from its arsenic group. In alkaline solutions, Lewisite may hydrolyze to form acetylene and sodium arsenate. Leonard and Lauwerys (1980) reviewed the carcinogenicity, teratogenicity and mutagenicity of a wide variety of arsenic compounds. Arsenic, as sodium arsenate or arsenite, is known to be embryotoxic and teratogenic in a number of animal species (Leonard and Lauwerys, 1980). In a comparison of Lewisite and sodium arsenite toxicity in the rabbit following intravenous administration, Inns et al. (1988) reported

that the LD<sub>50</sub> of sodium arsenite and Lewisite were not similar (7.6 and 1.8 mg/kg, respectively). Furthermore, significant differences in tissue arsenic content and pathology were reported for the two chemicals.

Very little information is available on the effects of Lewisite using *in vitro*, mammalian cell systems. However, the mutagenicity of arsenic compounds *in vitro* has been reviewed (Leonard and Louwerys, 1980). In general all of the arsenic compounds investigated in mammalian cell systems produced chromosomal aberrations. No information is available for mutation induction in mammalian systems, although arsenic compounds were analyzed in bacterial systems and some were mutagenic while others were not. We report here on the cytotoxicity, mutagenicity, and also the clastogenicity of Lewisite using Chinese hamster ovary (CHO) cells.

## MATERIALS AND METHODS

### Lewisite

#### Procurement and Characterization

A shipment of 25 ml of dichloro(2-chlorovinyl)arsine (Lewisite, Agent L) was received from the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) on 7 March 1985. The chemical (Lot No. L-U-4273-CTF-M) was prepared by distillation on 30 September 1984 at the Chemical Research and Development Center (CRDC). The agent was analyzed by nuclear magnetic resonance ( $^1\text{H}$ -1 and  $^{13}\text{C}$ -13; CRDC SOP No. 8-1-83-1, Annex F) at the Research Directorate, CRDC. Results of the analyses, expressed as calculated weight percent, were 95.8 and 4.0 for trans and cis isomers of dichloro(2-chlorovinyl)arsine, respectively, and 0.2 for unknown compounds.

The Lewisite was divided into two equal portions, pipetted into 30-ml Wheaton vials, sealed and stored in secondary unbreakable containers in refrigerator storage at  $-6^\circ\text{C}$ . To comply with Good Laboratory Practices requirements, PNL requested that USAMRICD retain an aliquot of this lot of Lewisite.

Lewisite was analyzed on 20 January 1986 to detect the presence of common impurities, such as Lewisite oxide and the cis-trans isomers of bis(2-chlorovinyl)chloroarsine and tris(2-chlorovinyl)arsine (Rosenblatt et al., 1975). Measurement of the ultraviolet absorption spectrum of the sample in isooctane revealed that the spectrum and the absorptivity of the material at 215 nm agreed with published values in the literature (Rewick, et al., 1986; Mohler and Sorge, 1939) and did not indicate the presence of ultraviolet-absorbing compounds other than Lewisite. This conclusion was supported by our results from gas-chromatographic analyses of the sample following derivatization with 2-mercaptoethanol.

#### Selection and Characterization of Diluent

Lewisite is relatively insoluble and also is rapidly hydrolyzed in water; therefore, absolute ethanol (EtOH) was employed as the diluent for dosing solutions in this study.

Lewisite in EtOH was assayed by gas chromatography, using a capillary column and flame-ionization detection. Lewisite was prepared by the addition of 2-mercaptoethanol; the reaction, which proceeds at room temperature, may be written:



In the procedure developed for the analysis, Lewisite samples were diluted 1:10 with isooctane prior to analysis. For the assay, 1.0 ml of the sample was diluted with 0.5 ml of a solution containing 112 ng of 1-chloronaphthalene and 5584 ng of 2-mercaptoethanol/ $\mu$ l in isooctane contained in a 1.5 ml automatic sampler vial with a Teflon-lined, crimped-top cap. The column (J&W Scientific, DB-5) temperature program was 90°C for 5 min (5°/min) to 140°C, 20°/min to 300°C and 300°C for 40 min. A Hewlett-Packard 5840A gas chromatograph and a 7672A automatic sample changer were used.

The results were within acceptable limits of analytical error for concentrations greater than 0.5 mg/ml. As the Lewisite concentrations in the solutions decreased, the assay results became less acceptable. The method was not sufficiently sensitive to detect concentrations of Lewisite below 0.1 mg/ml. Lewisite was stable in EtOH at concentrations above the detection limit for at least one day; all exposures were conducted within 24 hours of preparation of Lewisite solutions.

#### Chemicals Used

Dilutions of the Lewisite were made up in absolute (punctilious) ethanol (EtOH - U.S. Industrial Chemical Co.). A new bottle of ethanol was used for each experiment. Ethyl methanesulfonate (EMS - Sigma lot #95F-0226) was used as a positive control for mutation and sister chromatid exchange (SCE) studies. EMS proved inadequate as a positive control for aberration analysis and bleomycin (Sigma Lot #37F-0888) was substituted. All EMS dilutions were made up in absolute ethanol. 6-aminocryisine (6-AC: Aldrich lot #092797) was used as a positive control for materials that required S9 activation. 6-AC was solubilized in dimethylsulfoxide (DMSO: American Type Culture, Lot #129341). Metabolic activation was accomplished using Litton Bionetics rat

liver S9 preparation lot #07420. The 6-thioguanine (6-TG: Sigma lot #15f-4023), used as a selecting chemical in the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) mutation assay, was made up in sterile water as a 3 mM stock solution and used at a final concentration in medium of 30  $\mu$ M.

5-Bromo-2'-deoxyuridine (Brd Urd: Sigma lot #56F-0767 and #35F-0089), Hoechst dye (Sigma lot #106F-0458 and #25F-3538), and Giemsa stain (Gurr's improved R66 lot #772201) were used to differentiate sister chromatids. The Sorensen's buffer used in this technique was made up as a 10X solution (A and B stock). Stock A = 9.07g K H<sub>2</sub>PO<sub>4</sub> in 100 ml H<sub>2</sub>O; Stock B = 9.47 g Na<sub>2</sub>HPO<sub>4</sub> in 100 ml H<sub>2</sub>O. The final working solution was 10 ml A and 10 ml B brought up to 100 ml's total with dH<sub>2</sub>O and the pH was adjusted to 6.8.

### Cell Culture Media

F12 medium supplemented with 5% fetal bovine serum (fbs) was used for routine cell culture. F12 medium - hypoxanthine (-HX) supplemented with 5% dialyzed fetal bovine serum (dfbs) was used for mutant selection.

### Identification of Cell Line

The cells used in this study were designated CHO/C18 which was a subclone of CHO used for mutation analysis (Jostes et al., 1980). These cells have been subsequently maintained in liquid nitrogen and cultured in F12 medium supplemented with 5% fetal bovine serum. Cell identification was routinely verified using chromosome analysis. Chromosome analysis included showing that the mean chromosome number was 21 and that the karyotype was consistent with CHO cells.

### In Vitro Assays

One mutation assay and two cytogenetic assays were used in this study. The mutation assay utilized the HGPRT locus (6-thioguanine resistance). The two cytogenetic analyses were chromosomal aberration analysis (a measurement of chromosome damage) and SCE (a measurement of chromosome rearrangement). The experimental design for each was as follows:

## Mutation Analysis

In addition to the test compound, the CHO/HGPRT mutation assay contained the following elements; a positive control (EMS); a promutagen (6-AC) which required metabolic activation and a solvent control (EtOH) which served as a negative control. Three concentrations of the test compound were assayed in replicate or triplicate. CHO cells were treated in 75 cm<sup>2</sup> tissue culture flasks. Cells ( $0.5 - 1.0 \times 10^6$  per flask) were plated into 10 ml F12 medium with 5% fetal calf serum (fcs) and were incubated for 15-24 hours before exposure. The test compound was diluted in EtOH and a standard volume (50  $\mu$ l) was added to 10 ml of F12 medium minus serum for cell exposure. Direct-acting mutagens were added to 10 ml of F12 medium - fbs and incubated at 37°C for 1 hour + 5 min.

Rat liver microsomes (S9) were used in some experiments to activate promutagens. In this case the S9 and associated cofactors were added to the medium just before treatment.

After treatment cultures were washed 3 times with saline G and fresh F12 + 5% fbs was added to the cultures. The cells were then incubated for an additional 20-30 hours before trypsinization to alleviate possible trypsin effects. After trypsinization the cells were plated for initial survival (day 1) and for phenotypic expression (6-10 days). At the end of the expression period the cells were trypsinized and replated into F12 -Hx + 5% dfbs for determination of plating efficiency and  $5 \times 10^4$  cells/well were plated into 3, 6-well plates containing F12 -Hx, 30  $\mu$ M 6-TG and 5% dfbs for selection of mutant colonies.

After colony formation the plates were fixed, stained, and counted. The total number of mutant colonies and the plating efficiency was determined at each treatment. The mutation frequency was then calculated by dividing the total number of mutant colonies by the cells plated into 6-TG corrected for plating efficiency.

## Cytogenetic Analysis

Treatment protocols were as described previously for the mutation analysis. After treatment the cells were cultured for at least 24 hours in

F12 medium supplemented with 5% fbs for aberration analysis. If the chromosomes were to be scored for SCE, 10  $\mu$ M BrdUrd was present in the medium after treatment. After approximately 24 hours colcemid was added at a final concentration of 0.08  $\mu$ g/ml. Metaphase cells were collected by the "shake" method and the suspended cells were centrifuged, swelled, fixed and burst onto microscope slides. Cells were prepared for aberration analysis by staining in 5% Giemsa. Chromosomes were prepared for SCE analysis by a modification of the methodology of Perry and Wolff (1974).

### Statistical Analysis

Chromosome aberrations were scored using 100 metaphases per dose. In aberration studies each cell was evaluated as an individual treatment and means and standard errors are calculated within each experiment. Standard error is used because of the high number of naught values in each treatment and the poisson nature of aberration distribution. The standard error was determined by the formula: square root of the mean number of aberrations divided by the square root of the number of metaphases evaluated (Bradley et al. 1981).

SCE were scored and the data is expressed as SCE/cell. Analysis of 30 metaphases/treatment was made and significant differences were determined using the more conventional standard deviations (Remington, 1970).

Criteria for a positive mutation response were evaluated according to the genotox reports of Bradley et al., (1981). That is, any response 3 X the appropriate spontaneous value was taken to be positive.

## RESULTS

### Cytotoxicity

Figure 1 presents the CHO cell survival response (day 1) for 1 hour Lewisite exposures as determined by colony formation in two experiments. Using the data from experiment B a  $D_{57}$  of approximately  $0.5 \mu\text{M}$  Lewisite and an extrapolation number of 2.5 was derived. When the cells were exposed in the presence of S9 microsomal fractions, the survival was enhanced suggesting that the S9 itself interferes with Lewisite toxicity (Figure 2).

### Mutation Results

Initially, a dose range of  $0.12 \mu\text{M}$  to  $1.0 \mu\text{M}$  was selected for mutation analysis (Table 2, experiment A). This represents essentially the first decade of survival. Because the metabolic requirements of Lewisite are unknown S9 microsomal fractions were included in a replicate set of exposures. In this experiment the only mutation frequency that exceeded control values was at the highest exposure dose without S9 ( $1.0 \mu\text{M}$ ). Accordingly, a higher dose range of  $0.5$  to  $2.0 \mu\text{M}$  was selected for the second experiment (Table 2, experiment B). In this experiment the only value above spontaneous was the  $1.0 \mu\text{M}$  exposure with S9. In both cases in which the frequencies were above spontaneous values the variation of the control and treated frequencies suggest that they are not significantly different.

### Sister Chromatid Exchange

Table 3 presents data which show a small increase in chromosomal rearrangement (SCE) at all of the dose levels investigated with the exception of  $0.5 \mu\text{M}$  (experiment C) and  $0.25 \mu\text{M} + \text{S9}$  (experiment D). In no case however, were the treatment values significantly greater than the spontaneous values as determined by standard deviations. Furthermore, no value was greater than 56% above the spontaneous value nor was a concentration related increase observed over at least 3 concentrations.

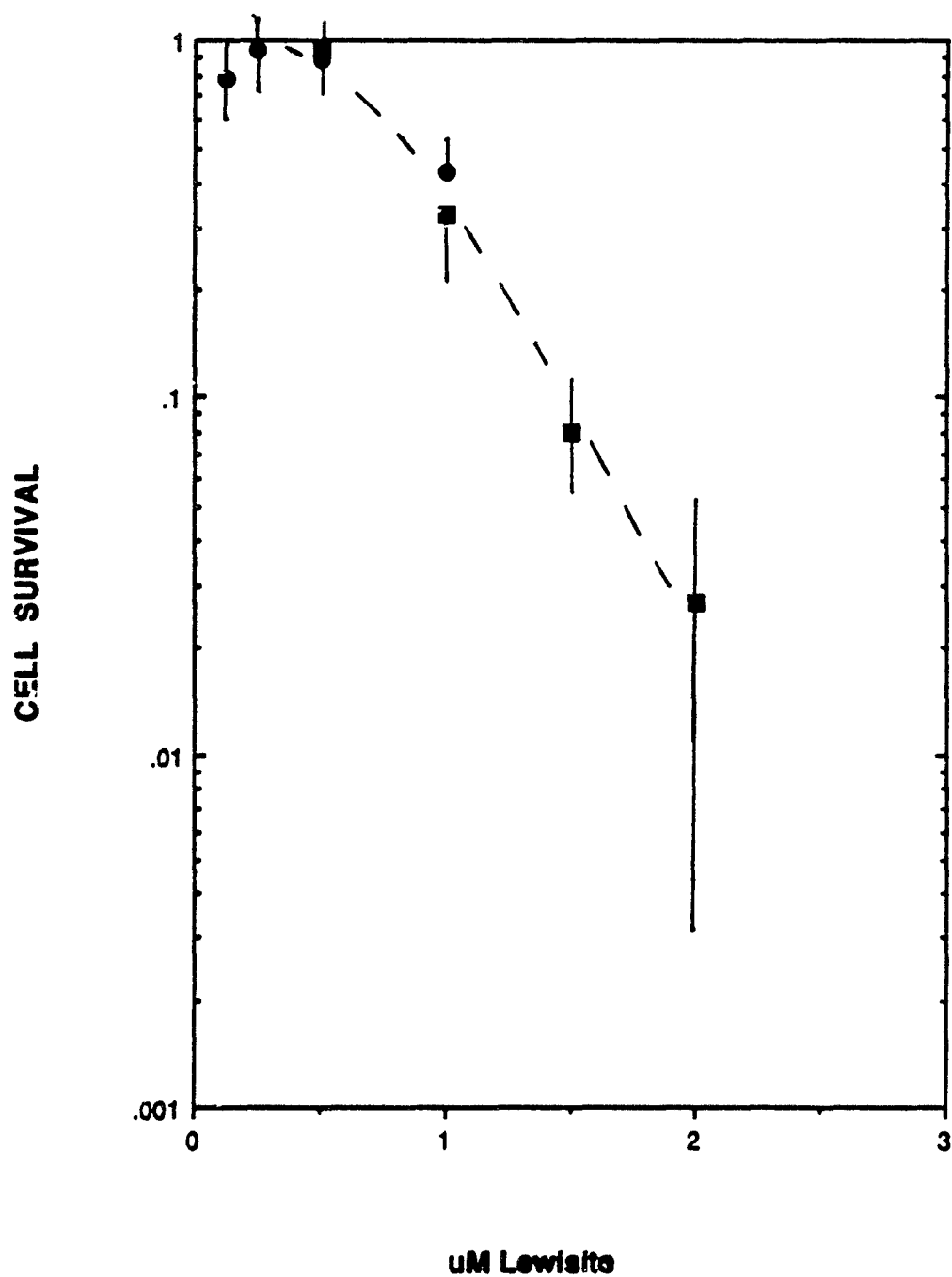


Figure 1. Fraction of cells surviving Lewisite treatment (day 1). Circles are from experiment A and squares are from experiment B. The line was drawn by eye to the data from experiment B.

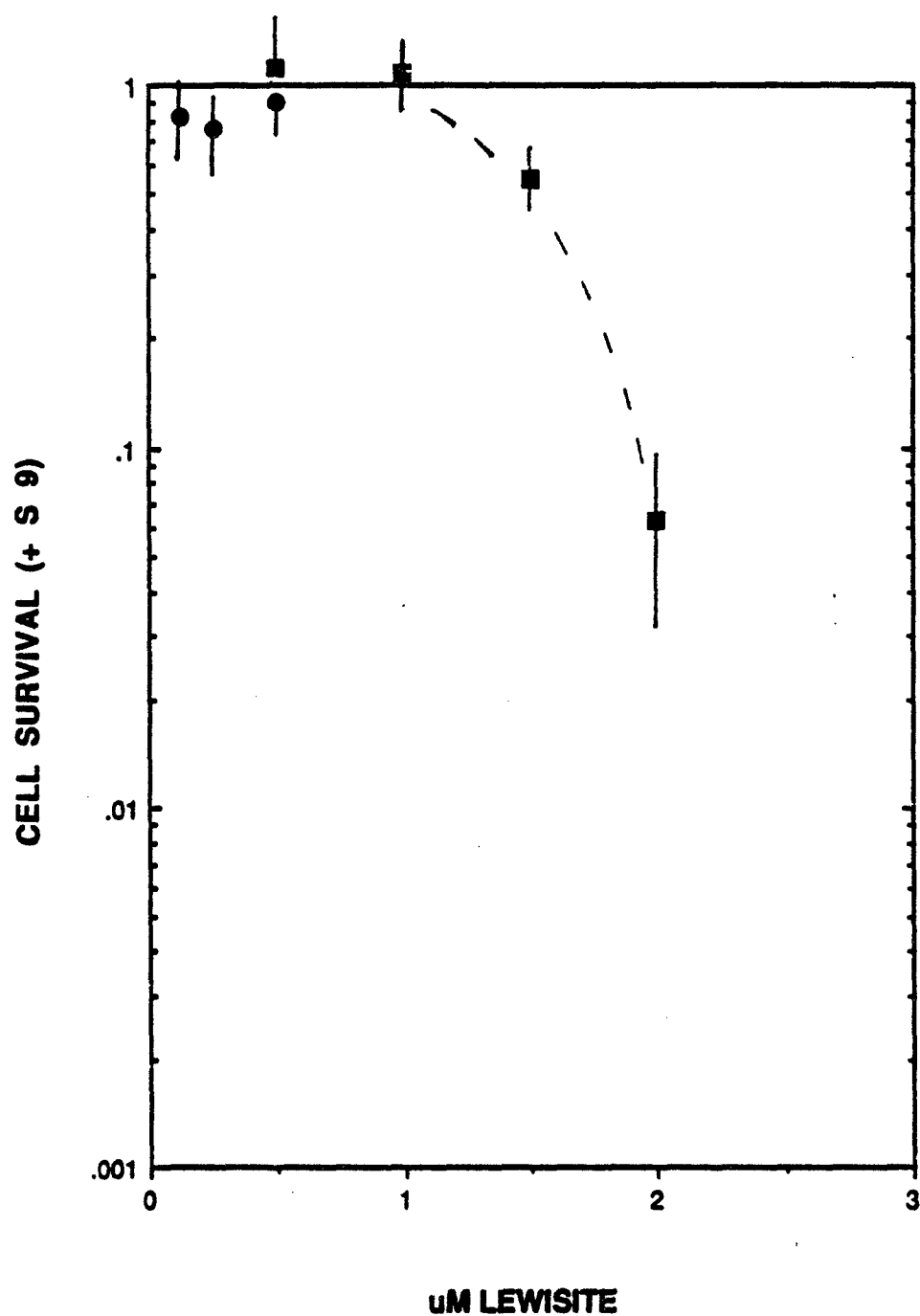


Figure 2. Fraction of cells surviving Lewisite treatment in the presence of S9 (day 1). Circles are experiment A and squares are experiment B. The line was drawn by eye to the data from experiment B. Note the increase in survival compared to that without S9 (see Figure 1).

Table 2. Lewisite (L) Induced Mutations at the HGPRT Locus in CHO Cells

Treatment	Mutations per Viable Cell x 10 <sup>-3</sup>					
	Experiment A			Experiment B		
	Mean	Induced <sup>a</sup>	Mean	Induced	Mean	Induced
EMS Control (1 mM)	208	(b)				
6AC Control (300 µM)	4.8		27	22 <sup>c</sup>	3.5	22
Control (EtOH)	8.4	(b)			5.1	19 <sup>c</sup>
0.12 µM L	0	0	3.8	0	(b)	(b)
0.25 µM L	0	0	1.9	0	(b)	(b)
0.50 µM L	7.2	0	5.6	0	2.3	0
1.0 µM L	13	4.6	5.1	0	0	12
1.5 µM L	(b)		(b)		0	0.7
2.0 µM L	(b)		(b)		0.8	1.8
					0	0

<sup>a</sup> Induced = Average L values less control frequency, except where noted for 6AC.

<sup>b</sup> Not done.

<sup>c</sup> Induced = (6AC + S9) - (6AC -S9).

Table 3. Lewisite (L) Induced Sister Chromatid Exchanges in CHO Cells

Treatment	SCE per Metaphase <sup>a</sup>					
	Experiment C			Experiment D		
	-S9 Mean $\pm$ SD	Induced <sup>b</sup>	+S9 Mean $\pm$ SD	-S9 Mean $\pm$ SD	Induced	+S9 Mean $\pm$ SD
EMS Control 1 mM	10.1 $\pm$ 3.4	4.2	(c)	(c)	(c)	(c)
150 $\mu$ M 6-AC	12.4 $\pm$ 4.6		16.8 $\pm$ 5.8	4.4 <sup>d</sup>	(c)	(c)
300 $\mu$ M 6-AC	(c)		(c)	9.0 $\pm$ 3.3	22.0 $\pm$ 7.0	13.0 <sup>d</sup>
Control (EtOH)	5.9 $\pm$ 2.0		5.2 $\pm$ 2.0	7.9 $\pm$ 3.3	6.9 $\pm$ 2.1	
0.25 $\mu$ M L	6.6 $\pm$ 2.7	0.7	7.4 $\pm$ 2.5	2.2	8.6 $\pm$ 2.7	0.7
0.50 $\mu$ M L	5.7 $\pm$ 1.5	0	7.0 $\pm$ 2.6	1.8	9.8 $\pm$ 3.2	1.9
0.75 $\mu$ M L	7.8 $\pm$ 3.4	1.9	8.1 $\pm$ 3.0	2.9	(e)	8.8 $\pm$ 2.6
1.00 $\mu$ M L	8.5 $\pm$ 2.9	2.6	6.5 $\pm$ 2.4	1.3	(e)	8.0 $\pm$ 3.3
					9.0 $\pm$ 0.4	2.1

<sup>a</sup> 30 Metaphases scored.

<sup>b</sup> Induced = Average L value less control frequency except where noted for 6AC.

<sup>c</sup> Not done.

<sup>d</sup> Induced = (6AC + S9) - (6AC - S9).

<sup>e</sup> Chromosome morphology inadequate for analysis.

## Clastogenic Effects

A dose range of 0.25 to 1.0  $\mu\text{M}$  Lewisite was selected for the aberration experiments based on survival results. This represents a survival range of 30 to 100 percent. Table 4 presents data from two experiments. In both experiments a threshold was seen above which aberration frequencies were significantly greater than control values. When cells were exposed to Lewisite in the presence of S9 (experiment E) a significant increase was not apparent. Because of this result and a similar inhibition of cytotoxicity (Figure 2) we did not include S9 in experiment F. The absolute toxicity (as judged by metaphase recovery and aberration induction) was greater in experiment F. Significant increases ( $P < 0.05$ ) in aberrations were observed at 0.5, 0.75, and 1.0  $\mu\text{M}$  Lewisite in experiment E and 0.5 and 0.75  $\mu\text{M}$  Lewisite in experiment F as judged by standard error.

Table 4. Lewisite (L) Induced Chromosome Aberrations in CHO Cells

Aberrations per Metaphase <sup>a</sup>									
Treatment	Replicate	Experiment E				Experiment F			
		-S9		+S9		-S9		+S9	
		Mean ± SEM	Induced <sup>b</sup>	Mean ± SEM	Induced	Mean ± SEM	Induced	Mean ± SEM	Induced
Bleomycin Control		0.19 ± 0.04		(c)		0.08 ± 0.03			
Control (EtOH)		0 ± 0		0.01 ± 0.01		0.04 ± 0.02			
0.25 μM L	A	0.01 ± 0.01	0.01	0.01 ± 0.01	0	0.01 ± 0.01			
	B	(c)		(c)		0.02 ± 0.01			
Mean						0.02		0	
0.50 μM L	A	0.02 ± 0.01	0.02	0 ± 0	0	0.19 ± 0.04			
	B	(c)				0.09 ± 0.03			
Mean						0.14		0.10	
0.75 μM L	A	0.04 ± 0.02	0.04	0.03 ± 0.02	0.02	0.68 ± 0.08			0.64
	B	(c)		(c)		(d)			
1.0 μM L	A	0.30 ± 0.07	0.30	0 ± 0	0	(d)			
	B	(d)		(c)		(d)			

<sup>a</sup> 100 metaphases scored unless otherwise noted.

<sup>b</sup> Induced = Average L value less control value.

<sup>c</sup> Not done.

<sup>d</sup> Not enough metaphases to count.

## DISCUSSION

Lewisite induced chromosomal aberrations at micromolar exposure levels. Conversely, SCE were not observed in statistically significant numbers. In this regard, Lewisite is similar to bleomycin, a radiomimetic, antineoplastic agent. One interpretation of this observation would be that the action of the two agents might be similar. We have noted in this report that significant levels of mutations are not observed at the HGPRT locus. It is tempting to suggest that Lewisite affects the DNA in such a way that all "hits" are lethal. It is known, however, that bleomycin is a weak mutagen at the HGPRT locus and a strong mutagen at the L5178Y TK locus. A popular interpretation of this observation is that lesions induced by bleomycin affect many loci (multilocus deletions, translocations) and that the first lethal gene lies close to the HGPRT gene but is further removed from the TK locus in L5178Y cells.

In any case, chromosomal aberrations are often lethal events and no doubt contribute to cell death after Lewisite exposure. Whether or not the agent is mutagenic may require investigations at other loci, such as the L5178Y TK system. In at least one gene situation, CHO - HGPRT, Lewisite is not mutagenic over the first decade of survival.

#### LITERATURE CITED

Auerbach, C. and J.M. Robson. 1947. Tests of chemical substances for mutagenic action. Proc. Royal Soc. of Edinburgh 62B: 284-291.

Bradley, M.O., B. Bhuyan, M.C. Francis, R. Langerbach, A. Peterson and E. Huberman 1981 Mutagenesis by chemical agents in V79 Chinese hamster cells: a review and analysis of the literature - A report of the gene-tox program. Mutat. Res. 87:81-142.

Cameron, G.R., H.M. Carleton and R.H.D. Short. 1946. Pathological changes induced by Lewisite and Allied Compounds. J. Pathol. Bacteriol. 58, 411-422.

Cassarett, L.J. and J. Doull. 1986. Toxicology. The Basic Science of Poisons, 3rd Ed., MacMillan Publishers, New York, NY.

Gates, M., J.W. Williams and J. A. Zapp. 1946. Aresenicals. Chemical Warfare Agents and Related Chemical Problems, Summary Technical Report of Division 9, National Defense Research Committee, Vol. 1, Parts I and II, pp. 83-114. Washington, D.C.

Hackett, P.L., L. B. Sasser, R.L. Rommereim, J.A. Cushing, R. L. Buschbom, and D. R. Kalkwarf. 1987. Teratology studies on Lewisite and sulfur mustard agents: Effects of Lewisite in rats and rabbits. AD-A187495. U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD.

Inns, R.H., J.E. Bright and T.C. Marrs. 1988. Comparative acute systemic toxicity of sodium arsenite and dichloro(2-chlorovinyl)arsine in rabbits. Toxicology 51: 213-222.

Jostes, R.F., K.M. Bushnell and W.C. Dewey 1980 X-ray induction of 8-azaguanine-resistant mutants in synchronous Chinese hamster ovary cells. Radiat. Res. 83:146-161.

Krause, H. and E.I. Grussendorf. 1978. Syntony of Bowen's disease and Lewisite scar. Hautarzt 29: 490-493.

Leonard, A. and R.R. Lauwerys. 1980. Carcinogenicity, teratogenicity and mutagenicity of arsenic. Mutat. Res. 75: 49-62.

Loveless, A. 1951. Qualitative aspects of the chemistry and biology of radiomimetic (mutagenic) substances. Nature 167: 338-342.

Mohler, H. and J. Sorge. 1939. Chemical warfare materials. XII. Light absorption by nose and throat, lung and skin poisons in ultraviolet of short wave length. Helv. Chim. Acta 22: 235-239.

National Academy of Science. 1977. Medical and Biologic Effects of Environmental Pollutants, Arsenic. Washington, D.C.

Perry, P. and S. Wolff. 1974. New Giemsa method for the differential staining of sister chromatids. Nature 251: 156-158.

Remington, R.D. 1970. Statistics with Application to the Biological and Health Sciences. 1970 Prentice Hall, Englewood Cliff, NJ.

Rewick, R.T., M.L. Schumacher and D. L. Haynes. 1986. The uv absorption spectra of chemical agents and stimulants. Appl. Spectroscopy 40: 152-156.

Rosenblatt, D.H., T.A. Miller, J.C. Dacre, I. Muul, and D.R. Cogley (eds.). 1975. Problem definition of potential environmental pollutants. II. Physical, chemical, toxicological and biological properties of 16 substances. In: U.S. Army Medical Bioengineering Research and Development Technical Report 7509. Fort Detrick, Frederick, MD.

Wada, S., Y. Nishimoto, M. Miyanishi, S. Kambe, and R.W. Miller. 1968. Mustard gas as a cause of respiratory neoplasia in man. The Lancet 7753: 1161-1163.

Yamakido, M. and T. Shigenobu. 1985. The causes of death in the retired workers of Okuno-Jima poison gas factory. Jpn. J. Med. 34: 311-322.

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Study Dates:

Initiated: 28 April 1987  
Completed: 15 September 1987

Data are property of the U.S. Army and will be archived under the Army's direction at approved facilities.

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R. F. Jostes, Jr.

10-31-87  
Date

## Toxicity Study of Lewisite in the Chinese Hamster and Ovary Cells

### Quality Assurance Statement

Listed below are the phases and/or procedures included in the study described in this report which were reviewed by the Quality Assurance Unit specifically for this study and the dates the reviews were performed and findings reported to management. (All findings were reported to the study director or his designee at the time of the review.)

Phase/Procedure Reviewed	Review Date	Date Findings Submitted in Writing to Study Director/Management
Cell Harvesting*	6/26/87 & 7/01/87	7/06/87
Cell Replating	6/26/87 & 7/01/87	7/06/87
Chromosome Dropping	9/03/87	9/22/87
Final Report	6/15-16/89 & 9/6-7/89	10/16/89

\* Cell dosing was conducted in the limited access chemical surety facility.

Patricia L. Rasmussen  
Quality Assurance Auditor

10/19/89  
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